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CLONING AND CHARACTERIZATION OF SARCO/ENDOPLASMIC RETICULUM Ca^{2+}-ATPase (SERCA) FROM CRAYFISH AXIAL MUSCLE

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Summary

The discontinuous pattern of muscle growth during the moulting cycle of a freshwater crustacean (the crayfish *Procambarus clarkii*) was used as a model system to examine the regulation of the expression of *S*arco/*E*ndoplasmic *R*eticulum *C*a^{2+}-*A*TPase (SERCA). We describe the cloning, sequencing and characterization of a novel SERCA cDNA (3856 bp) obtained from crayfish axial abdominal muscle by reverse transcription/polymerase chain reaction (RT-PCR) followed by rapid amplification of cDNA ends (RACE). This complete sequence contains a 145 base pair (bp) noncoding region at the 5′ end, a 3006 bp open reading frame coding for 1002 amino acid residues with a molecular mass of 110 kDa and 705 bp of untranslated region at the 3′ end. This enzyme contains all the conserved domains found in 'P'-type ATPases, and the hydropathy profile suggests a transmembrane organization typical of other SERCAs. It exhibits 80 % amino acid identity with *Drosophila melanogaster* SERCA, 79 % identity with *Artemia franciscana* SERCA, 72 % identity with rabbit fast-twitch muscle neonatal isoform SERCA1b, 71 % identity with slow-twitch muscle isoform SERCA2 and 67 % identity with SERCA3. Sequence alignment revealed that regions anchoring the cytoplasmic domain in the membrane were highly conserved and that most differences were in the NH_2 terminus, the central loop region and the COOH terminus. Northern analysis of total RNA from crayfish tissues probed with the 460 bp fragment initially isolated showed four bands (7.6, 7.0, 5.8 and 4.5 kilobases) displaying tissue-specific expression. SERCA was most abundant in muscle (axial abdominal, cardiac and stomach), where it is involved in Ca^{2+} resequestration during relaxation, and in eggs, where it may be implicated in early embryogenesis. The level of SERCA mRNA expression in axial abdominal muscle varied during the moulting cycle as determined by slot–blot analysis. SERCA expression was greatest during intermoult and decreased to approximately 50 % of this level during pre- and postmoult. Patterns of gene expression for SERCA and other sarcomeric proteins during the crustacean moulting cycle may be regulated by ecdysteroids and/or mechanical stimulation.

Key words: muscle, cDNA sequence, mRNA expression, tissue-specific distribution, moulting cycle, sarco/endoplasmic reticulum Ca^{2+}-ATPase, Crustacea, *Procambarus clarkii*.

Introduction

Subcellular Ca^{2+} homeostasis is critical to all eukaryotic cells. A primary Ca^{2+}-translocating protein is the Ca^{2+}-ATPase that transports Ca^{2+} against its electrochemical gradient using free energy from the hydrolysis of ATP. Ca^{2+} 'pumps' are located on the intracellular membranes of the sarco- or endoplasmic reticulum (*S*arco/*E*ndoplasmic *R*eticulum *C*a^{2+}-*A*TPase, SERCA) and on the plasma membrane (*P*lasma *M*embrane *C*a^{2+}-*A*TPase, PMCA). SERCA removes Ca^{2+} from the cytosol by sequestering it in the sarco/endoplasmic reticulum (SR/ER); PMCA extrudes Ca^{2+} from the cell. Regulating intracellular Ca^{2+} concentration in the range 100–200 nmol l^{-1} requires coordination between SERCA and PMCA, especially during net transepithelial Ca^{2+} flux. A question of fundamental importance is whether expression of the Ca^{2+} pump is regulated in response to Ca^{2+}-pumping activity and, if so, by what molecular mechanism. It is also important to understand the molecular diversity and differential tissue-specific expression of these ancient gene families.

In mammals, SERCA was initially cloned and sequenced from muscle, where it is highly abundant and functions to resequester Ca^{2+} during relaxation (MacLennan et al., 1985; Brandl et al., 1986, 1987). The less abundant PMCA was cloned 3 years later (Shull and Greeb, 1988; Verma et al., 1988). Both are integral membrane proteins of 1000 amino acid residues with three cytoplasmic domains (an ATP-binding site, a phosphorylation site and a transduction domain; Brandl et al., 1986) joined to a set of 10 transmembrane α-helices by a narrow pentahelical stalk of α-helices. ATP bound to one cytoplasmic domain phosphorylates an adjoining aspartate, inducing Ca^{2+} translocation. Mutagenesis studies have

identified the following residues as Ca^{2+}-binding amino acid residues and their corresponding transmembrane helices as the Ca^{2+}-binding and translocation domain: Glu309 in transmembrane helix M4; Glu771 in M5; and Asn796, Thr799 and Asp800 in M6 (Clarke et al., 1989).

Mammalian SERCA is encoded by a family of three homologous genes which, through alternative splicing, encode five isoforms (MacLennan et al., 1985; Wu and Lytton, 1993). The SERCA1 gene is expressed as a neonatal or adult isoform in fast-twitch skeletal muscle (Brandl et al., 1986, 1987). The SERCA2 gene generates a SERCA2a muscle-specific form expressed in slow-twitch skeletal, cardiac and smooth muscle and a SERCA2b isoform expressed ubiquitously (Gunteski-Hamblin et al., 1988; Lytton and MacLennan, 1988; Lytton et al., 1989; Wu et al., 1995). The SERCA3 gene (Burk et al., 1989) is expressed in endothelial and epithelial cells of a variety of tissues. SERCA genes have also been cloned from other vertebrate phyla including birds (Karin et al., 1989; Campbell et al., 1991), frogs (Vilsen and Andersen, 1992) and fish (Tullis and Block, 1996). In mammals, expression of SERCA is regulated by changing levels of thyroid hormone (Rohrer and Dillmann, 1988), chronic muscle stimulation (Briggs et al., 1990) or cardiac hypertrophy (Nagai et al., 1989), all of which alter Ca^{2+}-pumping activity.

Invertebrate SERCAs have been cloned from two arthropods, the fruit fly *Drosophila melanogaster* (Magyar and Varadi, 1990) and the brine shrimp *Artemia franciscana* (Palmero and Sastre, 1989), and PMCA has been cloned from *Paramecium tetraurelia* (Elwess and van Houten, 1997). A single SERCA gene has been identified in *Drosophila melanogaster* (Varadi et al., 1989; Magyar et al., 1995). In *Artemia franciscana*, a single gene transcript originates two SERCA mRNAs, by alternative splicing (4.5 and 5.2 kb; Palmero and Sastre, 1989), that code for protein isoforms homologous to vertebrate SERCA2a and SERCA2b (Escalante and Sastre, 1993) and that are developmentally regulated.

As arthropods, crustaceans exhibit intermittent growth resulting in discontinuous patterns of muscle growth and transepithelial Ca^{2+} flux. The crustacean moulting cycle has emerged as a model system for the regulation of expression of genes encoding sarcomeric proteins (Mykles, 1997) because of location-dependent variation in muscle synthesis. To allow removal through the narrow basi-ischial joint, claw muscles undergo a programmed premoult atrophy not seen in other somatic muscles such as leg/abdominal muscles (Mykles and Skinner, 1990). Subsequently, the muscles undergo longitudinal growth during immediate postmoult, with cross-sectional growth occurring in later postmoult/intermoult. In our laboratory, we employ the natural crustacean moulting cycle of the freshwater crayfish *Procambarus clarkii* to study discontinuous Ca^{2+} homeostasis (Wheatly, 1999). Premoult demineralization of the calcified exoskeleton results in a negative Ca^{2+} balance. Following cuticular shedding (ecdysis), Ca^{2+} is taken up from the external water at rates of 2–10 mmol kg^{-1} h^{-1} (positive Ca^{2+} balance) and used to mineralize the new cuticle. Crayfish have a long evolutionary history in fresh water, which is Ca^{2+}-limited ($[Ca^{2+}]$<1 mmol l^{-1}). During intermoult, they regulate extracellular $[Ca^{2+}]$ hyperionically; the kidney (antennal gland) assists by producing dilute urine. During postmoult, net Ca^{2+} influx across polarized cells (branchial, renal and digestive epithelia) typically involves a combination of Ca^{2+} channels and energy-dependent processes (PMCA and a Na^+/Ca^{2+} exchanger) on the plasma membrane; in addition, SERCA maintains cytoplasmic $[Ca^{2+}]$ in the face of transcellular flux. This model lends itself to the study of the regulation of the expression of Ca^{2+}-translocating proteins.

In the present article, we describe the cloning and characterization of crayfish (*Procambarus clarkii*) axial abdominal muscle SERCA cDNA, its tissue-specific distribution and differential expression as a function of the moulting cycle.

Materials and methods

Animal material

Crayfish *Procambarus clarkii* (Girard) were obtained from Carolina Biological Supply and maintained in 40 l aquaria in filtered aerated water at room temperature (21 °C). Tissues were removed from animals at various stages of the natural moulting cycle. Premoult status was determined from the gastrolith index (McWhinnie, 1962). Postmoult status was classified with reference to the day of ecdysis (shedding). Following decerebration, the following tissues were dissected out: axial abdominal muscle, gill, antennal gland, hepatopancreas (liver), stomach, heart and unfertilized eggs.

Isolation of total RNA and mRNA

After dissection, tissues were frozen immediately in liquid nitrogen and stored at −80 °C. Total RNA was isolated by acid guanidinium thiocyanate–phenol–chloroform extraction (Chomczynski and Sacchi, 1987). Messenger RNA was separated from total RNA using an oligothymidylic acid [oligo$(dT)_{18}$] cellulose column (Stratagene; Sambrook et al., 1989). RNA or mRNA was quantified spectrophotometrically at wavelengths of 260 and 280 nm. Only RNAs with an absorbance ratio (A_{260}:A_{280}) of greater than 1.8 were used for further experiments. The integrity of RNA was confirmed on a 0.72 mol l^{-1} formaldehyde/1 % agarose denaturing gel run in Mops buffer (5 mmol l^{-1} sodium acetate, 1 mmol l^{-1} EDTA, 20 mmol l^{-1} Mops, pH 6.6).

Amplification of central 460 bp fragment by RT-PCR

First-strand cDNA was reverse-transcribed from 400 ng of axial abdominal muscle mRNA using the SuperScript II RNase H reverse transcriptase (Gibco BRL) with oligo$(dT)_{12–18}$ as primer. On the basis of two highly conserved regions of the published *Artemia* muscle SERCA sequence (corresponding to nucleotides 2176–2195 and 2617–2636; Palmero and Sastre, 1989), two non-degenerate primers 1A (5′-GAAATTTCCGCTATGACTGG-3′ sense) and 1B (5′-ACAGTGGCAGCACCAACATA-3′ antisense) were

designed using Oligo 4.0 software (American Biotechnology Laboratory). These primers targeted a fragment of approximately 460 bp located between the 5′-(*p*-fluorosulphonyl)benzoyladenosine (FSBA)-binding site and transmembrane region 7 of a typical SERCA. Polymerase chain reactions (total volume 50 µl) included 2 µl of first-strand cDNA reaction, 20 mmol l^{-1} Tris-HCl (pH 8.4), 50 mmol l^{-1} KCl, 1.5 mmol l^{-1} $MgCl_2$, 0.2 mmol l^{-1} dNTP mix and 0.2 µmol l^{-1} of each primer. Polymerase chain reaction (PCR) was performed by the hot-start method using 2.5 units of *Taq* DNA polymerase (Gibco BRL) or *pfu* DNA polymerase (Stratagene) in an MJ Research thermal cycler. PCR started with denaturation of cDNA at 94 °C for 3 min followed by 25 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min and a final cycle at 72 °C for 7 min. Negative controls in which reactions contained only one primer or no template cDNA were included. PCR products were analyzed by electrophoresis on a 0.8 % to 1.0 % agarose gel with 0.5 µg ml^{-1} of ethidium bromide in 1× TAE buffer (40 mmol l^{-1} Tris, 40 mmol l^{-1} sodium acetate and 1 mmol l^{-1} EDTA). The DNA bands were visualized under ultraviolet light.

Marathon rapid amplification of cDNA ends (RACE)

Rapid amplification of cDNA ends (RACE) was employed to complete the 3′ and 5′ regions of the muscle SERCA. To obtain cDNA for amplification of the 3′ and 5′ regions of muscle SERCA, double-stranded cDNA was obtained using a Marathon cDNA amplification kit (Clontech). Briefly, mRNA was reverse-transcribed by MMLV reverse transcriptase using a modified lock-docking oligo(dT) primer. Following the creation of blunt ends with T_4 DNA polymerase, the double-stranded cDNA was ligated to the Marathon cDNA adaptor, which has the complementary sequence to the Clontech adaptor primer (AP1). PCR amplification of the 5′ region was performed on the resulting template using AP1 and the gene-specific primer RACE2 (5′-TCAGCTTTCTTCAGGGCAGGTGCATC-3′, antisense), which was designed on the basis of the 460 bp partial cDNA fragment of crayfish muscle SERCA. PCR amplification of the 3′ region was carried out using the gene-specific primer RACE1 (5′-GATGCACCTGCCCTGAAGAAAGCTGA-3′, sense) and the Clontech adaptor primer (AP1). PCR conditions were as follows: one cycle at 94 °C for 3 min; five cycles at 94 °C for 30 s, 72 °C for 4 min; five cycles at 94 °C for 30 s, 70 °C for 4 min; 25 cycles at 94 °C for 30 s, 68 °C for 4 min followed by one cycle at 72 °C for 5 min. PCR products were ligated to pCR 2.1 vector (Invitrogen) for transformation into INVαF host cells (Invitrogen). Each clone was digested with the appropriate restriction enzymes and subcloned for sequencing. At least two independent clones were sequenced. Whenever necessary, a third clone was sequenced to correct for sequence discrepancies in the first two clones.

DNA sequencing

The cDNA clones were sequenced by the dideoxynucleotide method using Sequenase 2.0 (Amersham Life Science) or by automated sequencing using the Pharmacia ALF (Wayne State University Macromolecular Core Facility, Detroit, MI, USA).

DNA and amino acid sequence analysis

The complete sequence was analyzed with MacDNASIS software (Hitachi). Sequence homology was revealed through a GenBank data base search using the BLAST algorithm (Altschul et al., 1990). Hydropathy analysis was performed with MacDNASIS software (Kyte and Doolittle, 1982).

Northern blot analysis

Northern blot analysis was performed to determine the distribution of SERCA in intermoult tissue. Total RNA (15 µg) from axial abdominal muscle, heart, eggs, stomach, hepatopancreas, antennal gland and gills was fractionated by electrophoresis through 0.72 mol l^{-1} formaldehyde/1 % agarose denaturing gel and transferred overnight to a Schleicher & Schuell Nytran Plus membrane by capillary elution in 10× SSC (1× SSC is 150 mmol l^{-1} NaCl, 15 mmol l^{-1} sodium citrate). RNA was fixed by ultraviolet crosslinking using a UVC-515 ultraviolet multilinker from Ultra-Lum (120 mJ cm^{-2}). RNA molecular mass markers (a 0.24–9.5 kb ladder) were run along with the samples, then visualized under ultraviolet light after staining with ethidium bromide. The membranes were prehybridized with 6× SSC, 2× Denhardt's reagent (0.4 g Ficoll type 400, 0.4 g polyvinylpyrrolidone, 0.4 g bovine serum albumin in 1 l H_2O) and 0.1 % SDS for 4 h at 68 °C. Hybridization was performed overnight at 68 °C in the prehybridization solution with 40 ng of the ^{32}P-labelled 460 bp partial sequence corresponding to crayfish muscle SERCA

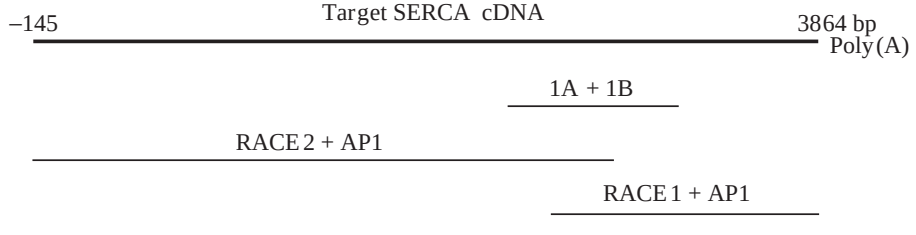
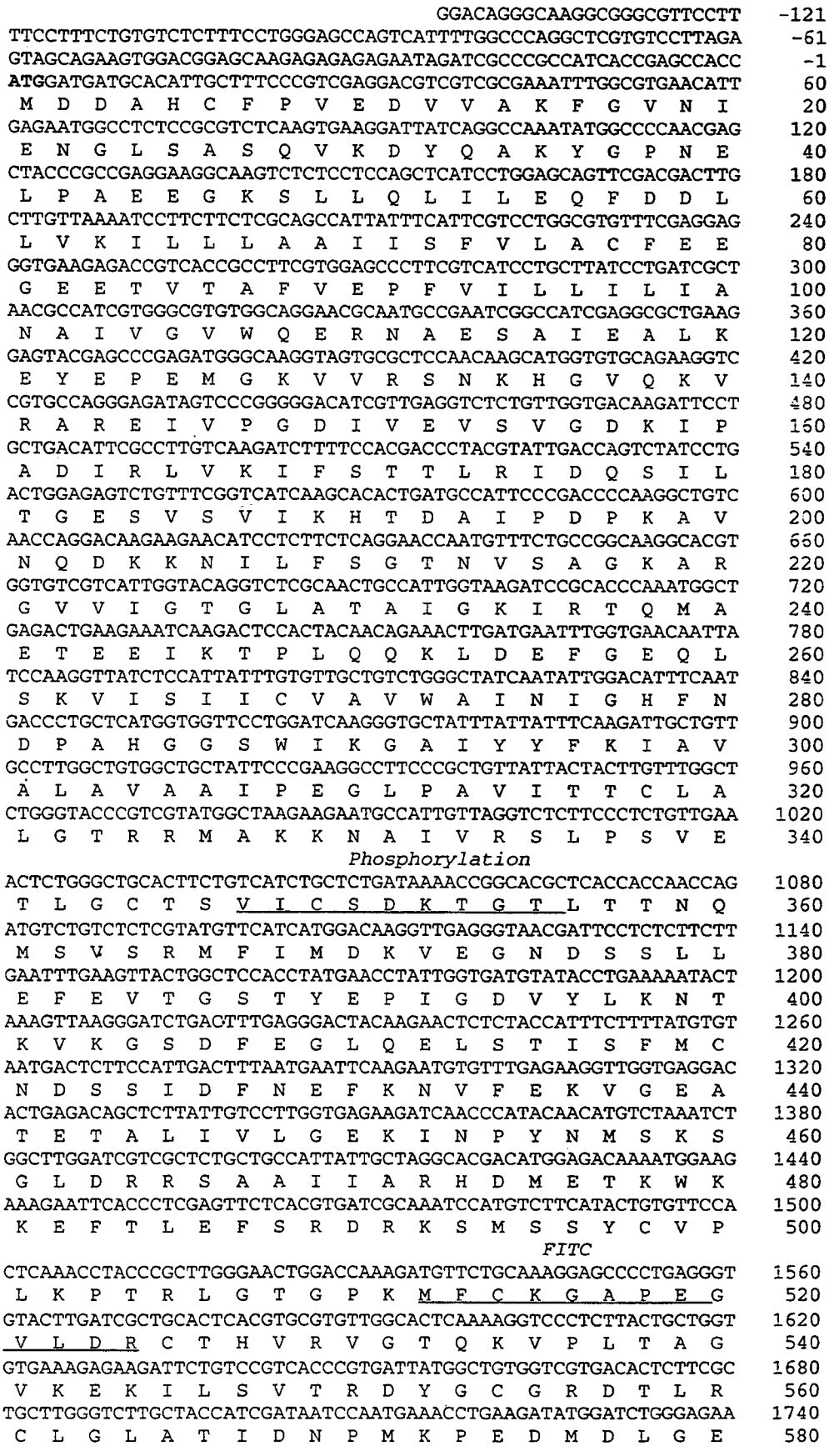


Fig. 1. Strategy for cloning the complete cDNA sequence of *S*arco/*E*ndoplasmic *R*eticulum *C*a^{2+}-*A*TPase (SERCA) from crayfish axial abdominal muscle. Double-stranded cDNA from axial abdominal muscle was prepared and ligated to an adaptor (Clontech) and used as template for polymerase chain reaction (PCR). Initially, two primers, 1A and 1B, were used to produce (by PCR) a central 460 bp partial DNA sequence. Two gene-specific primers, RACE1 and RACE2, along with AP1 (an adaptor primer supplied by Clontech) were used to perform 5′ rapid amplification of cDNA ends (RACE) and 3′ RACE to obtain the complete DNA sequence.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TTCCTTTCTGTGTCTCTTTCCTGGGAGCCAGTCATTTTGGCCCAGGCTCGTGTCCTTAGA -61
GTAGCAGAAGTGGACGGAGCAAGAGAGAGAGAATAGATCGCCCGCCATCACCGAGCCACC -1
ATGGATGATGCACATTGCTTTCCCGTCGAGGACGTCGTCGCGAAATTTGGCGTGAACATT 60
M D D A H C F P V E D V V A K F G V N I 20
GAGAATGGCCTCTCCGCGTCTCAAGTGAAGGATTATCAGGCCAAATATGGCCCCAACGAG 120
E N G L S A S Q V K D Y Q A K Y G P N E 40
CTACCCGCCGAGGAAGGCAAGTCTCTCCTCCAGCTCATCCTGGAGCAGTTCGACGACTTG 180
L P A E E G K S L L Q L I L E Q F D D L 60
CTTGTTAAAATCCTTCTTCTCGCAGCCATTATTTCATTCGTCCTGGCGTGTTTCGAGGAG 240
L V K I L L L A A I I S F V L A C F E E 80
GGTGAAGAGACCGTCACCGCCTTCGTGGAGCCCTTCGTCATCCTGCTTATCCTGATCGCT 300
G E E T V T A F V E P F V I L L I L I A 100
AACGCCATCGTGGGCGTGTGGCAGGAACGCAATGCCGAATCGGCCATCGAGGCGCTGAAG 360
N A I V G V W Q E R N A E S A I E A L K 120
GAGTACGAGCCCGAGATGGGCAAGGTAGTGCGCTCCAACAAGCATGGTGTGCAGAAGGTC 420
E Y E P E M G K V V R S N K H G V Q K V 140
CGTGCCAGGGAGATAGTCCCGGGGGACATCGTTGAGGTCTCTGTTGGTGACAAGATTCCT 480
R A R E I V P G D I V E V S V G D K I P 160
GCTGACATTCGCCTTGTCAAGATCTTTTCCACGACCCTACGTATTGACCAGTCTATCCTG 540
A D I R L V K I F S T T L R I D Q S I L 180
ACTGGAGAGTCTGTTTCGGTCATCAAGCACACTGATGCCATTCCCGACCCCAAGGCTGTC 600
T G E S V S V I K H T D A I P D P K A V 200
AACCAGGACAAGAAGAACATCCTCTTCTCAGGAACCAATGTTTCTGCCGGCAAGGCACGT 660
N Q D K K N I L F S G T N V S A G K A R 220
GGTGTCGTCATTGGTACAGGTCTCGCAACTGCCATTGGTAAGATCCGCACCCAAATGGCT 720
G V V I G T G L A T A I G K I R T Q M A 240
GAGACTGAAGAAATCAAGACTCCACTACAACAGAAACTTGATGAATTTGGTGAACAATTA 780
E T E E I K T P L Q Q K L D E F G E Q L 260
TCCAAGGTTATCTCCATTATTTGTGTTGCTGTCTGGGCTATCAATATTGGACATTTCAAT 840
S K V I S I I C V A V W A I N I G H F N 280
GACCCTGCTCATGGTGGTTCCTGGATCAAGGGTGCTATTTATTATTTCAAGATTGCTGTT 900
D P A H G G S W I K G A I Y Y F K I A V 300
GCCTTGGCTGTGGCTGCTATTCCCGAAGGCCTTCCCGCTGTTATTACTACTTGTTTGGCT 960
A L A V A A I P E G L P A V I T T C L A 320
CTGGGTACCCGTCGTATGGCTAAGAAGAATGCCATTGTTAGGTCTCTTCCCTCTGTTGAA 1020
L G T R R M A K K N A I V R S L P S V E 340
Phosphorylation
ACTCTGGGCTGCACTTCTGTCATCTGCTCTGATAAAACCGGCACGCTCACCACCAACCAG 1080
T L G C T S V I C S D K T G T L T T N Q 360
ATGTCTGTCTCTCGTATGTTCATCATGGACAAGGTTGAGGGTAACGATTCCTCTCTTCTT 1140
M S V S R M F I M D K V E G N D S S L L 380
GAATTTGAAGTTACTGGCTCCACCTATGAACCTATTGGTGATGTATACCTGAAAAATACT 1200
E F E V T G S T Y E P I G D V Y L K N T 400
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K V K G S D F E G L Q E L S T I S F M C 420
AATGACTCTTCCATTGACTTTAATGAATTCAAGAATGTGTTTGAGAAGGTTGGTGAGGAC 1320
N D S S I D F N E F K N V F E K V G E A 440
ACTGAGACAGCTCTTATTGTCCTTGGTGAGAAGATCAACCCATACAACATGTCTAAATCT 1380
T E T A L I V L G E K I N P Y N M S K S 460
GGCTTGGATCGTCGCTCTGCTGCCATTATTGCTAGGCACGACATGGAGACAAAATGGAAG 1440
G L D R R S A A I I A R H D M E T K W K 480
AAAGAATTCACCCTCGAGTTCTCACGTGATCGCAAATCCATGTCTTCATACTGTGTTCCA 1500
K E F T L E F S R D R K S M S S Y C V P 500
FITC
CTCAAACCTACCCGCTTGGGAACTGGACCAAAGATGTTCTGCAAAGGAGCCCCTGAGGGT 1560
L K P T R L G T G P K M F C K G A P E G 520
GTACTTGATCGCTGCACTCACGTGCGTGTTGGCACTCAAAAGGTCCCTCTTACTGCTGGT 1620
V L D R C T H V R V G T Q K V P L T A G 540
GTGAAAGAGAAGATTCTGTCCGTCACCCGTGATTATGGCTGTGGTCGTGACACTCTTCGC 1680
V K E K I L S V T R D Y G C G R D T L R 560
TGCTTGGGTCTTGCTACCATCGATAATCCAATGAAACCTGAAGATATGGATCTGGGAGAA 1740
C L G L A T I D N P M K P E D M D L G E 580

Fig. 2

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GCTTCTAAGTTCTATACATATGAAGTTAATATGACATTTGTTGGCGTAGTTGGTATGCTT   1800
 A  S  K  F  Y  T  Y  E  V  N  M  T  F  V  G  V  V  G  M  L     600
GACCCACCACGTAAGGAAGTTAAAGATTCAATCCAGAGATGTCGTGATGCTGGTATCCGT  1860
 D  P  P  R  K  E  V  K  D  S  I  Q  R  C  R  D  A  G  I  R     620
GTTATTGTCATTACTGGAGACAATAAGGCAACTGCTGAGGCTATCTGCCGTCGTATTGGA  1920
 V  I  V  I  T  G  D  N  K  A  T  A  E  A  I  C  R  R  I  G     640
GTTTTTAAAGAAGATGAAGATACAACTGGTATGTCATATTCTGGCCGTGAGTTTGACGAG  1980
 V  F  K  E  D  E  D  T  T  G  M  S  Y  S  G  R  E  F  D  E     660
CTAAGTCCTGAAGAACAGAGGCAGGCATGTATTCGTTCCCGCCTCTTCTCTCGTGTAGAG  2040
 L  S  P  E  E  Q  R  Q  A  C  I  R  S  R  L  F  S  R  V  E     680
CCCTTCCATAAGTCAAAGATTGTTGAATATCTTCAAGGAGAGAACGAGATCTCAGCCATG  2100
 P  F  H  K  S  K  I  V  E  Y  L  Q  G  E  N  E  I  S  A  M     700
          FSBA/CIRATP
ACAGGTGATGGTGTGAATGATGCACCTGCCCTGAAGAAAGCTGAAATTGGCATTGCTATG  2160
 T  G  D  G  V  N  D  A  P  A  L  K  K  A  E  I  G  I  A  M     720
GGATCTGGTACTGCTGTGGCCAAGTCTGCCTCTGAAATGGTGCTGGCTGATGACAACTTC  2220
 G  S  G  T  A  V  A  K  S  A  S  E  M  V  L  A  D  D  N  F     740
TCCTCTATTGTGGCTGCTGTTGAAGAAGGTCGTGCTATTTACAACAACATGAAGCAGTTC  2280
 S  S  I  V  A  A  V  E  E  G  R  A  I  Y  N  N  M  K  Q  F     760
ATCCGTTACCTCATTTCTTCCAATGTTGGTGAGGTTGTTTCCATCTTTTTGACTGCTGCT  2340
 I  R  Y  L  I  S  S  N  V  G  E  V  V  S  I  F  L  T  A  A     780
CTAGGTCTTCCAGAAGCTCTTATCCCAGTCCAGCTCCTGTGGGTCAACCTTGTAACTGAT  2400
 L  G  L  P  E  A  L  I  P  V  Q  L  L  W  V  N  L  V  T  D     800
GGCTTGCCTGCTACTGCCTTGGGCTTCAACCCTCCAGATCTTGATATTATGGACAAACCT  2460
 G  L  P  A  T  A  L  G  F  N  P  P  D  L  D  I  M  D  K  P     820
CCCCGCAGAGCTGACGAGTCCCTCATCTCTGGCTGGCTATTCTTCCGTTACATGGCCATT  2520
 P  R  R  A  D  E  S  L  I  S  G  W  L  F  F  R  Y  M  A  I     840
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 G  G  Y  V  G  A  A  T  V  F  A  A  S  W  W  F  M  Y  D  P     860
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 T  G  P  H  L  N  Y  Y  Q  L  S  H  H  L  Q  C  L  G  D  P     880
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 E  N  F  E  G  L  D  C  N  I  F  S  H  P  A  P  M  T  M  A     900
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 S  L  L  I  M  P  P  W  V  N  F  W  L  L  A  A  M  A  L  S     940
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 M  T  L  H  F  I  I  L  Y  I  D  I  L  S  T  V  F  Q  V  M     960
CCACTGTCTGTTGCCCAGTGGGTTGCTGTCTTGAAGATTTCCTTCCCTGTATTGCTGCTG  2940
 P  L  S  V  A  Q  W  V  A  V  L  K  I  S  F  P  V  L  L  L     980
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 Q  W  ***                                                      1002
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TTTGATTCCCTTGTTAAAATAAACTGTTAGCCAAGCTACTCTTGGGCAAACATTACCAAA  3540
AGTCGCACAAAGTCTGTTGTGTCCTGTGTTCGTGCTCGTGTCAATTCGCCCTTCAGAGAG  3600
CAATGAACGACCATACAAACATCCACTTGTTCAAGTACAAAGATGTGGTGCAATTTTGTA  3660
CCTCATTTTGTACATGCCACTCATTTATTGTTGATAGAATAAATAATCAAAAGTA(n)     3715
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Fig. 2. The complete nucleotide and deduced amino acid sequence of crayfish axial abdominal muscle *S*arco/*E*ndoplasmic *R*eticulum Ca^{2+}-*A*TPase (SERCA) cDNA. Nucleotides and amino acids are numbered to the right of the sequence. The phosphorylation site, the fluorescein isothiocyanate (FITC)-site and the 5′-*p*-fluorosulphonylbenzoyladenosine (FSBA)/γ-[4-(*N*-2-chloroethyl-*N*-methylamino)]benzylamide ATP (CIRATP) binding site are underlined and labelled. The start codon and the stop codon are indicated in bold letters. This sequence has been accepted by GenBank (accession number AF025849). The asterisks indicate the stop codon.

nucleotides 2086–2546. The fragment was purified using QIAquick gel extraction kit (Qiagen) and labelled for 3 h with [α-^{32}P]dATP to a specific activity of 1×10^9 cts min^{-1} μg^{-1} using a random-primer labelling kit (Gibco BRL). The labelled probe was then separated from unincorporated nucleotides by chromatography on a Sephadex G-50 Nick column (Pharmacia Biotech). Following high-stringency washes (four washes for 15 min at 60 °C in 0.1× SSC and 0.1 % SDS), membranes were exposed to X-ray film with intensifying screens at –80 °C. To confirm equal loading between samples, 18S RNA was quantified on a corresponding formaldehyde/agarose gel. Total RNA content was determined by measuring A_{260} and visualized by ethidium bromide staining.

Slot–blot analysis

To quantify the expression of SERCA in axial abdominal muscle during the moulting cycle, 5 μg of muscle total RNA was prepared from six individual crayfish at each stage of the moulting cycle (intermoult, late premoult and 1–2 days postmoult). The total RNAs were denatured for 15 min at 65 °C in a solution containing 50 % formamide, 7 % formaldehyde and 1× SSC (Sambrook et al., 1989). The samples were quickly cooled on ice, mixed with 2 volumes of 20× SSC and directly blotted onto a Schleicher & Schuell Nytran Plus membrane under gentle vacuum suction using a Bio-Dot SF microfiltration apparatus (Bio-Rad). The membrane was hybridized with the 460 bp muscle fragment probe under the stringent conditions described above for the northern blot. The hybridization signals were scanned using a laser densitometer (Zenith). The bound probe was stripped from the membrane by immersion twice for 15 min in boiling 0.05× SSC, 0.01 mol l^{-1} EDTA and 0.1 % SDS (Sambrook et al., 1989). To quantify the amount of RNA loaded in each slot, the membrane was rehybridized with oligo(dT)$_{18}$ (Harrison and El Haj, 1994). The oligo(dT)$_{18}$ (100 pmol) was 5′-end-labelled with [γ^{32}P]ATP and purified through Sephadex G-25 column (Pharmacia Biotech). The probe was diluted 10-fold with cold oligo(dT)$_{18}$ before use. The membranes were prehybridized for 3 h at room temperature in 6× SSC, 0.5 % SDS, 1× Denhardt's solution, 100 μg ml^{-1} denatured sonicated salmon sperm DNA and 0.05 % sodium pyrophosphate. Hybridization was carried out overnight under the same conditions except that 20 μg ml^{-1} tRNA was substituted for the salmon sperm DNA. The membrane was washed in 6× SSC, 0.1 % SDS, for 1 h at room temperature and in 3× SSC, 0.05 % SDS for 20 min at 32 °C. The hybridization signal was scanned again using the laser densitometer. The relative expression of SERCA mRNA was determined by calculating the ratio of the intensities of the images produced by the binding of the 460 bp probe and oligo(dT)$_{18}$ for each slot. Statistical significance compared with intermoult expression was evaluated by *t*-test (Dowdy and Wearden, 1991). An experiment was carried out to verify that oligo(dT)$_{18}$ binds exclusively to mRNA under the experimental conditions described. Total intermoult muscle RNA (320 μg) was extracted twice with 0.5 g of oligo(dT)$_{18}$ cellulose (Stratagene) to remove mRNA. The slot–blot experiment was repeated using the remaining RNA as control. Total muscle RNA (5 μg) and control RNA (total RNA minus mRNA, 5 μg) were used to run slot–blots under identical hybridization and wash conditions. The results (not shown) revealed that oligo(dT)$_{18}$ hybridized strongly with the total RNA but that there was negligible hybridization after mRNA had been removed. This confirms that the oligo(dT)$_{18}$ binds with the mRNA and that there is no cross reaction with rRNA.

Results

Primers 1A and 1B were successful in amplification of a discrete product from crayfish axial abdominal muscle cDNA. The size of the PCR product was 460 bp, matching the expected size on the basis of the *Artemia* SERCA sequence. A search of GenBank confirmed that the nucleotide sequence matched exclusively with SERCAs from *Drosophila* (80 %), *Artemia* (73 %) and many other vertebrates. The amino acid sequence showed 85 % homology with *Artemia* and *Drosophila* and 82 % with rabbit fast-twitch muscle SERCA (1a or 1b). This partial sequence provided crucial DNA sequence information required for 5′ and 3′ RACE cloning of the complete SERCA cDNA. On the basis of the 460 bp partial sequence, two gene-specific primers, RACE 1 and RACE 2, were used together with Clontech's adaptor primers to perform Marathon RACE of crayfish axial abdominal muscle cDNA. The cloning strategy is depicted in Fig. 1. A major band of 1.7 kb was obtained from 3′ RACE and cloned into PCR 2.1 vector (Invitrogen). A major band of 2.2 kb was cloned into PCR 2.1 from 5′ RACE.

The complete nucleotide sequence and deduced amino acid sequence of crayfish axial abdominal muscle SERCA is shown in Fig. 2. This 3856 nucleotide sequence contains an open reading frame of 3006 bp coding for 1002 amino acid residues with a molecular mass of 110 360 kDa. The 5′-terminal 145 bp noncoding sequence is GC-rich (57 %). The initiator Met codon is part of a longer sequence, –CCACCATGG–, that contains a purine at position –3 and a G at position +4, both of which are necessary for efficient initiation of translation (Kozak, 1984). There is a 705 bp noncoding sequence at the 3′ end before the poly(A) region.

The predicted amino acid sequence displays structural features common to other SERCA pumps. The hydropathy profile of crayfish axial abdominal muscle SERCA (Fig. 3) reveals 10 distinct regions of hydrophobicity representing sections of the amino acid sequence that are buried in the lipid bilayer of the membrane. It appears that four of these putative transmembrane domains are located near the N-terminal region and the remaining six are located near the C terminus, with a large cytoplasmic loop in between. The pattern of the transmembrane arrangement is very similar to that of *Artemia* or mammalian SERCA1 and SERCA2.

A search of the GenBank database using the BLAST algorithm (Altschul et al., 1990) revealed 29 high-score matches with the putative crayfish axial abdominal muscle SERCA amino acid sequence. These matches were

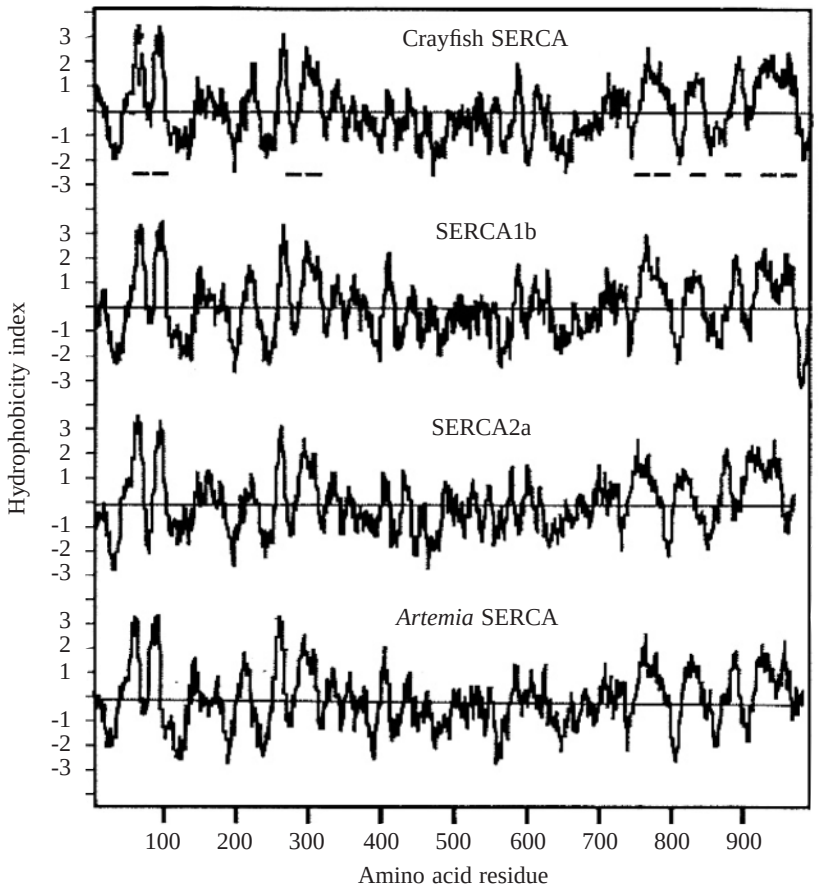

Fig. 3. Hydropathy plot of crayfish axial abdominal muscle *S*arco/*E*ndoplasmic *R*eticulum *C*a^{2+}-*A*TPase (SERCA) in comparison with that of rabbit SERCA1b (Brandl et al., 1986) and SERCA2a (Lytton et al., 1989) and *Artemia franciscana* SERCA (Palmero and Sastre, 1989). Hydrophobicity values were determined by the method of Kyte and Doolittle (1982) using a window of 12 residues (MacDNASIS, Rainbow Technologies, Inc.). Putative transmembrane segments are indicated by bold horizontal bar lines.

exclusively SERCA sequences from a variety of invertebrates and vertebrates (Brandl et al., 1986; Karin et al., 1989; Lytton et al., 1989; Palmero and Sastre, 1989; Magyar and Varadi, 1990; Wu and Lytton, 1993). The deduced amino acid sequence of crayfish SERCA shares highest homology with *Drosophila* SERCA (Magyar and Varadi, 1990), with 80 % identity. Compared with *Artemia* SERCA (Palmero and Sastre, 1989), although there is a 79 % identity between the amino acid sequences, the sequence alignment has gaps at residues 81 and 369. Comparing the crayfish SERCA amino acid sequence with three vertebrate SERCA isogenes (Fig. 4), the crayfish sequence is 72 % identical with rabbit fast-twitch muscle neonatal isoform SERCA1b (Brandl et al., 1986). The crayfish sequence shares 71 % amino acid identity with slow-twitch muscle isoform SERCA2 (Lytton et al., 1989) with a gap introduced between residues 510 and 511. There is only 67 % amino acid sequence homology with SERCA3 (Burk et al., 1989).

The tissue distribution of this crayfish SERCA gene was examined using a northern blot of total RNA from various crayfish tissues probed with the 460 bp fragment initially isolated (Fig. 5). Four bands were detected with molecular masses of 7.6, 7.0, 5.8 and 4.5 kb. The 4.5 kb isoform is specific to axial abdominal muscle, where it is expressed in high quantity. This isoform is probably the template for the cloned cDNA. The difference in molecular mass is due mainly to two factors. First, the 5′ upstream noncoding region may be much longer than the cloned sequence of 145 bp. Second, the 3′ end poly(A) tail often extends to several hundred bases that are not included as part of the 3856 bp cDNA fragment. A secondary band of 7.0 kb was also observed in axial muscle. The same northern blot exposed for only 2 h revealed that the 7.5 kb species is barely detectable compared with the 4.5 kb species (data not shown). The same probe recognized a prominent 5.8 kb isoform in heart and stomach. A 7.6 kb band that was predominant in egg was also observed in heart and stomach. Under the experimental conditions for this northern blot, bands were not detected in the hepatopancreas, antennal gland and gills, although we have preliminary evidence from northern analysis of mRNA that it is expressed in low abundance in each of these tissues (see Discussion). Equal loading of samples was confirmed by the 18S RNA at 1.9 kb (in crustaceans, total RNA gels typically reveal an intense 18S RNA and a less-intense 28S RNA).

Slot–blot analysis was used to quantify axial abdominal muscle SERCA mRNA expression level at different stages of the moulting cycle. The relative expression of axial abdominal muscle SERCA was determined by the ratio of SERCA mRNA to total mRNA in each slot. This is to compensate for inter-sample variation in each sample preparation (Harrison and El Haj, 1994). The RNA slot–blot experiment (Fig. 6) showed that the relative expression of SERCA mRNA during premoult

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Crayfish     MDDAHCFPVEDVVAKFGVNIENGLSASQVKDYQAKYGPNELPAEEGKSLLQLILEQFDDL      60
Artemia      .E...AKKW.E..DY...DP.R..ALE...KN.E..............T.........
Rabbit FT    .EA..SKST.ECL.Y...SETT..TPD...RHLE...H..........WE.VI...E..
Rabbit ST    .EN..TKT..E.LGH....EST...LE...KLKE.W.S.........T..E.VI...E..

                      M1                            M2
Crayfish     LVKILLLAAIISFVLACFEEG   EETVTAFVEPFVILLILIANAIVGVWQERNAESAIE      117
Artemia      ...........L...L...HDDEA.QL..Y.............V......K.......
Rabbit FT    ..R......C......W....   ...I............................N...
Rabbit ST    ..R......C......W....   DD.I............V..............N...

Crayfish     ALKEYEPEMGKVVRSNKHGVQKVRAREIVPGDIVEVSVGDKIPADIRLVKIFSTTLRIDQ      177
Artemia      ...........I.AD.T.I..IK..DL.....  I.........L..IS.L........
Rabbit FT    ...........Y.ADRKS..RIK..D.........A....V.....ILS.K.....V..
Rabbit ST    ...........Y.QDRKS..RIK.KD........IA....V......TS.K.....V..

Crayfish     SILTGESVSVIKHTDAIPDPKAVNQDKKNILFSGTNVSAGKARGVVIGTGLATAIGKIRT      237
Artemia      ..............PV...R........M..............M....N....S...
Rabbit FT    ..............EPV...R........M......IA....L.I.AT..VS.E.....D
Rabbit ST    ..............PV...R........M......IA....M...VA..VN.E.....D

                                        M3
Crayfish     QMAETEEIKTPLQQKLDEFGEQLSKVISIICVAVWAINIGHFNDPAHGGSWIKGAIYYFK      297
Artemia      ..F....M...................V.............................
Rabbit FT    ...A..QD...................L......L.........V......R.......
Rabbit ST    E.VA..QER..................L..I...I.........V......R.......

                      M4                                                Phos
Crayfish     IAVALAVAAIPEGLPAVITTCLALGTRRMAKKNAIVRSLPSVETLGCTSVICSDKTGTLT      357
Artemia      ............................................................
Rabbit FT    ............................................................
Rabbit ST    ............................................................

Crayfish     TNQMSVSRMFIMDKVEGNDSS LLEFEVTGSTYEPIGDVYLKNTKVKGSDFEGLQELSTI      416
Artemia      ..........VFKDIPDDAAPE.YQ..L.........ETFMQGQ.INAA.YDAVK.IT..
Rabbit FT    ......CK...I...D.DFC. .N..SI.....A.E.E.LKNDKPIRSGQ.D..V..A..
Rabbit ST    ......C....L...D.ETC. .N..TI.....A...E.HKDDKP..CHQTD..V..A..

Crayfish     SFMCNDSSIDFNEFKNVFEKVGEATETALIVLGEKINPYNMSKSGLDRRSAAIIARHDME      476
Artemia      CM.....A.....Y.QA.................L....L..A.K......LVV.E..D
Rabbit FT    CAL.....L....T.G.Y...........TT.V..M.VF.TEVRN.SKVER.NACNSVIR
Rabbit ST    CAL....AL.Y..A.G.Y...........TC.V..M.VFDTELK..SKIER.NACNSVIK

                                                        FITC
Crayfish     TKWKKEFTLEFSRDRKSMSSYCVPLKPTRLGTGPKMFCKGAPEGVLDRCTHVRVGTQKVP      536
Artemia      .R.......................AGL.SN.....V..................K...
Rabbit FT    QLM.............V..S.A.SS.AAV.N...V.......I...NY.....TR..
Rabbit ST    QLM.............V..T.N.TS..TSMS...V.......I.....I...ST...

Crayfish     LTVGVKEKILSVTRDYGCGRDTLRCLGLATIDNPMKPEDMDLGEASKFYTYEVNMTFVGV      596
Artemia      M.PAIMD...E...A..T........A.....D..D.K...IIDST..VK..Q.C.....
Rabbit FT    M.GP........IKEW.T........A...R.T.P.R.E.V.DDS.R.ME..TDL.....
Rabbit       M.....Q..M..I.EW.S.S......A...H...LRR.E.H.KDSAN.IK..T.L....C

Crayfish     VGMLDPPRKEVKDSIQRCRDAGIRVIVITGDNKATAEAICRRIGVFKEDEDTTGMSYSGR      656
Artemia      ...........L.A.E...A.........................G...N.E..A.T..
Rabbit FT    ...........MG...L.........M......G..I.......I.G.N.EVADRA.T..
Rabbit ST    ........I..AS.VKL..Q......M......G..V.......I.GQE..V.AKAFT..

                                                    FSBA/CIRATP
Crayfish     EFDELSPEEQRQACIRSRLFSRVEPFHKSKIVEYLQGENEISAMTGDGVNDAPALKKAEI      716
Artemia      ...D..V.G..D.VA.....A................MG.....................
Rabbit FT    ...D.PLA...E..R.ACC.A....S..........SYD..T..................
Rabbit ST    .....N.SA..D..LNA.C.A....S.......F..SFD..T..................

                                                                  M5
Crayfish     GIAMGSGTAVAKSASEMVLADDNFSSIVAAVEEGRAIYNNMKQFIRYLISSNVGEVVSIF      776
Artemia      .............A..........T.........................I.......
Rabbit FT    ............T............T..............................C..
Rabbit ST    ............T............T..............................C..
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Fig.4

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                                    M6
Crayfish    LTAALGLPEALIPVQLLWVNLVTDGLPATALGFNPPDLDIMDKPPRRADESLISGWLFFR      836
Artemia     ....................................N........G..T......
Rabbit FT   ....................................R...SPK.P.........
Rabbit ST   ......F.............................N....NPK.P.........

                 M7
Crayfish    YMAIGGYVGAATVFAASWWFMYDPTGPHLNYYQLSHHLQCLGDPENFEGLDCNIFSHPAP      896
Artemia     .....T.......G..AH...MS....G..F.........TPEN.Y...I..E...D.H.
Rabbit FT   ............G..A.....AED..GVT.H..T.FM..TE.HPH......E..EA.E.
Rabbit ST   .L...C.......G..A...IAADG..RVSF.....F...KE.NPD...V..A..ES.Y.

                      M8                                        M9
Crayfish    MTMALSVLVTIEMLNALNSLSENQSLLIMPPWVNFWLLAAMALSMTLHFIILYIDILSTV      956
Artemia     ................I.........V....S.I..IS.IC.......V...VE.....
Rabbit FT   ............C.............MR......I...GSIC...S...L...V.P.PMI
Rabbit ST   ............C.............MR....E.I..VGSIC...S...L...VEP.PLI

                         M10
Crayfish    FQVMPLSVAQWVAVLKISFPVLLLDETLKFIARNYTDVPEQIKQQW                    1002
Artemia     ..IC..TLTE.IV.............V...V..K...EFSF..
Rabbit FT   .KLKA.DLT..LM.....L..IG...I........LED..DERRK
Rabbit ST   ..IT..N.T..LM.....L..I.M......V....LEPAILE
```

Fig. 4. Comparison of the deduced amino acid sequence of crayfish axial abdominal muscle *S*arco/*E*ndoplasmic *R*eticulum *Ca*$^{2+}$-*A*TPase (SERCA) with that of *Artemia franciscana* muscle (Palmero and Sastre, 1989), rabbit fast-twitch (FT) skeletal muscle SERCA1 (Brandl et al., 1986) and rabbit slow-twitch (ST) skeletal muscle SERCA2 (Brandl et al., 1986). Residues from *Artemia franciscana* and rabbit SERCA1 and 2 that are identical to those of crayfish SERCA are indicated by dots. A three-space gap is introduced into crayfish and rabbit SERCA sequences following residue 81 and a single gap is introduced following residue 378 to maintain the alignment. Crayfish axial abdominal muscle SERCA amino acids are numbered on the right. Hydrophobic regions that may span the membrane are underlined and labelled M1–M10. The phosphorylation (Phos), fluorescein isothiocyanate (FITC) and the 5′-*p*-fluorosulfonylbenzoyladenosine/γ-[4-(*N*-2-chloroethyl-*N*-methylamino)] benzylamide ATP binding sites (FSBA/CIRATP) are underlined.

and postmoult decreased respectively to 49 % (average from two experiments, N=6, P<0.005) and 47 % (average from two experiments, N=6, P<0.01) of intermoult expression. Although two isoforms are expressed in muscle (Fig. 5), the 4.5 kb species is predominantly expressed. Therefore, the difference in expression of SERCA during the moulting cycle can be attributed mainly to the 4.5 kb isoform.

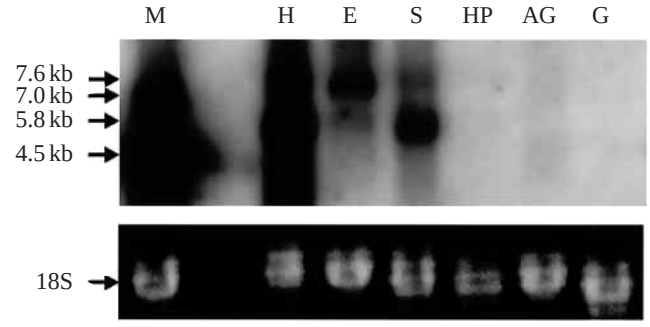

Fig. 5. (Top) Northern blot analysis of *S*arco/*E*ndoplasmic *R*eticulum *Ca*$^{2+}$-*A*TPase (SERCA) isoforms in intermoult crayfish tissues. Total RNA (15 μg) from axial abdominal muscle (M), heart (H), eggs (E), stomach (S), hepatopancreas (HP), antennal gland (AG) and gill (G) were loaded in each lane. The membrane was hybridized to a 460 bp probe (residues 2086–2546 of the crayfish muscle SERCA coding region) and exposed to X-ray film for 24 h. (Bottom) 18S RNA concentration on corresponding formaldehyde/agarose gel before being transferred to the membrane to serve as control (A_{260}; visualized by ethidium bromide staining).

Discussion

This study presents for the first time the cloning and molecular characterization of SERCA specific to axial abdominal muscle of crayfish *Procambarus clarkii*, including its tissue distribution and expression during the moulting cycle. Prior to this study, there were only two reports of SERCA characterization in arthropods, one in *Drosophila melanogaster* (Magyar and Varadi, 1990) and the other in *Artemia franciscana* (Palmero and Sastre, 1989). Both studies were performed on whole-body preparations because of the small size of the organisms.

The crayfish axial abdominal SERCA gene encodes a protein of 1002 amino acid residues with molecular mass of 110 kDa. The deduced amino acid sequence reveals that it contains all the conserved functional domains in P-type ATPases, such as the sequence ICSDKTGTLT (residues 348–357), in which the Asp351 residue serves as phosphorylation site (Post and Kume, 1973), the fluorescein isothiocyanate (FITC)-binding site MFCKGAVLD (512–523) and the peptides that bind ATP analogues FSBA NEISAMTGDGVNDAPALKK (695–713) (Ohta et al., 1986) and CIR-ATP (Ovchinnikov et al., 1987). Crucial charged and polar residues (Glu309, Glu771, Asn796, Thr799 and Asp800), believed to form high-affinity Ca^{2+}-binding sites in SERCA (Maruyama and MacLennan, 1988; Clarke et al., 1989), are also conserved. A pentapeptide motif KILLL (residues 63–67), believed to act as a signal for retention of the enzyme in the ER/SR, is present (Anderson et al., 1983). The hydropathy

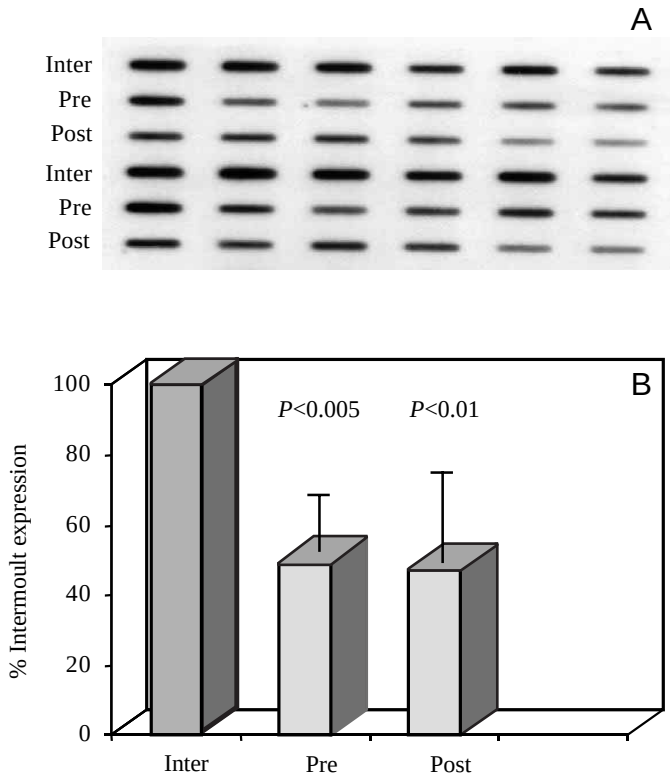

Fig. 6. (A) Slot–blot analysis of *S*arco/*E*ndoplasmic *R*eticulum *C*a^{2+}-*A*TPase (SERCA) mRNA in axial abdominal muscle during different stages of the moulting cycle. Muscle total RNA (5 μg) from 18 crayfish at different moult stages was used [intermoult (*N*=6), late premoult (*N*=6) and postmoult (1–2 days, *N*=6); two repetitions]. The membrane was treated as for a northern blot and exposed to X-ray film for 1 day. (B) Quantification of axial abdominal muscle SERCA mRNA during moult stages. Values [ratio of SERCA mRNA/poly(A) RNA] are expressed as a percentage of the intermoult control value. Values are the mean ± S.E.M. of two repetitions. Inter, intermoult; Pre, premoult; Post, postmoult.

profile of crayfish axial abdominal muscle SERCA shows similarity with the transmembrane organization of SERCA1 and SERCA2 from rat and with SERCA from *Artemia* (Fig. 3), suggesting a common protein tertiary structure within the membrane lipid bilayer. Briefly, there are two transmembrane segments in the NH_2-terminal region (M1 and M2) followed by a small cytosolic loop between the membrane-traversing stretches M3 and M4. A large cytoplasmic domain lies between M4 and M5, then precedes six transmembrane segments in the COOH-terminal part of the polypeptide.

When the amino acid sequence is aligned with known SERCAs from *Artemia* (Palmero and Sastre, 1989) or with rabbit fast-twitch skeletal SERCA1 or rabbit slow-twitch skeletal muscle SERCA2 (Brandl et al., 1986), the differences are concentrated at the NH_2 terminus, the central loop region and the COOH terminus (Fig. 4). Two regions that are highly conserved among the species are residues 247–363 and 699–822; both showed greater than 96 % amino acid identity. These are the polypeptide regions that ‘anchor’ the cytoplasmic domain to the membrane and are thought to be important for energy transduction. It is interesting to observe that unlike SERCA1a, a vertebrate skeletal muscle adult isoform, the crayfish SERCA has an extended COOH terminus with several quite hydrophilic amino acid residues VPEQIKQQY. This resembles the COOH terminus of vertebrate neonatal fast-twitch muscle SERCA1b. This may indicate that, before the two phyla diverged 600 million years ago, they shared the same ancestral SERCA gene with a charged COOH terminus. The vertebrates evolved to acquire an alternatively spliced adult isoform, SERCA1a.

Although this crayfish SERCA clone was isolated from axial abdominal muscle, northern analysis (Fig. 5) revealed that the gene is broadly expressed in several tissue types with four possible isoforms. The 4.5 kb species is specific to and highly abundant in muscle. A second muscle-specific isoform at 7.0 kb is less strongly expressed. In heart and stomach, two SERCA isoforms exhibit a similar pattern of expression, namely a predominant 5.8 kb isoform and a less-abundant 7.6 kb isoform. The same two isoforms are expressed in eggs; however, in this tissue, the 7.6 kb isoform is predominant and the 5.8 kb is less abundant. The high levels of expression of SERCA in muscle are consistent with its physiological role in Ca^{2+} sequestration during muscular relaxation (MacLennan et al., 1985; Brandl et al., 1986, 1987; Gunteski-Hamblin et al., 1988; Lytton and MacLennan, 1988). The oocyte stores many mRNA sequences that are utilized in different stages of embryonic development, and high levels of expression of SERCA in eggs indicate the obvious importance of the gene in early embryogenesis. On the basis of northern analysis of total RNA, this SERCA was not detected in the hepatopancreas, antennal gland or gill under the conditions described in the present study. However, when northern analysis was repeated on mRNA (10 μg per lane), the probe hybridized with three isoforms (4.5, 7.6 and 10.1 kb) in each tissue and an additional 5.8 kb isoform in hepatopancreas (Z. Zhang, D. Chen and M. G. Wheatly, unpublished). In summary, preliminary data suggest that SERCA is expressed ubiquitously in crustacean tissues.

In *Artemia*, a single SERCA gene codes for two isoforms (Escalante and Sastre, 1993, 1994), whereas in *Drosophila* there is a single SERCA gene and gene product (Varadi et al., 1989; Magyar and Varadi, 1990; Magyar et al., 1995). The existence of four isoforms in crayfish makes the picture more complex. In a separate study, an isoform specific to crayfish cardiac muscle has been identified (D. Chen, Z. Zhang and M. G. Wheatly, unpublished results). While both muscle types are striated, the cardiac muscle would not be expected to undergo the intermittent growth exhibited by somatic muscles. Furthermore, cardiac muscle exhibits a different pattern of contractility, with continuous rhythmic contraction. The expression of the 7.6 kb SERCA mRNA in crayfish eggs decreases dramatically in adult tissues, except in the heart, suggesting developmental regulation. Since no data are available at the genomic DNA level, the mechanism of splicing cannot be elucidated.

In the present, study slot–blotting indicated that axial abdominal muscle SERCA was differentially expressed during the natural moulting cycle of the freshwater crayfish. The level of SERCA mRNA was greatest during the intermoult stage and decreased in the pre- and postmoult stages (Fig. 6) as a result of reduced gene transcription or reduced stability of the mRNA. To date, regulation of muscle gene expression during the crustacean moulting cycle has focused on the sarcomeric proteins actin and myosin, which exhibit muscle-specific patterns of gene expression. Levels of actin mRNA expression during the moulting cycle varied between claw and leg muscle in crayfish (El Haj et al., 1992), reflecting these different growth patterns. In leg muscle, the level of actin mRNA was elevated during pre- and postmoult, suggesting that actin is synthesized during late premoult and that transcription continues into postmoult. However, in the claw muscle, the greatest elevation in actin mRNA level occurs in early premoult, with levels falling immediately prior to ecdysis and increasing again during postmoult. Because of the inconstancy of actin expression during the moulting cycle, it is not a suitable housekeeping gene for the purposes of quantifying mRNA expression using northern analysis (M. G. Wheatly, unpublished results; D. W. Towle, personal communication).

Factors identified that may regulate expression of muscle genes include hormones (especially the ecdysteroid 20-hydroxyecdysone, 20-HE) and mechanical stimulation. Elevation of rates of synthesis of protein and actin in leg muscle during premoult coincided with a peak in haemolymph ecdysteroid titre (El Haj et al., 1992; Whiteley et al., 1992); however, expression remained elevated into the postmoult period despite recovery of 20-HE to intermoult levels. Meanwhile, cheliped muscle actin expression is inversely related to 20-HE level since it is greatest during postmoult, a time when 20-HE level is lowest. A possible explanation for differential sarcomeric protein expression is that ecdysteroid receptor expression/activation may vary between muscle groups as well as moult stages. For example, the ecdysteroid-responsive gene HHR3 was upregulated in lobster leg and abdominal muscle during premoult, whereas in the eyestalk it had its highest level of expression during intermoult and expression decreased during premoult (exhibiting the same trend as SERCA in the present study; El Haj et al., 1997). *In vitro* attempts to increase mRNA expression in intermoult leg muscles through exposure to premoult concentrations of 20-HE have met with mixed success (Whiteley et al., 1992; El Haj et al., 1996). This suggests that the steroid may promote hypersensitive sites that increase access for stage- and tissue-specific transcription factors or, to put it simply, that the ecdysteroid receptors may not be accessible until the premoult stage.

Mechanical stimulation may also regulate translation and protein modifications for the assembly of sarcomeric proteins during the moulting cycle. Typically, somatic muscles are stretched during postmoult following water uptake for skeletal expansion. Flexion of the lobster carpopodite extensor muscle has been shown to upregulate actin mRNA expression and myofibrillar growth (Harrison and El Haj, 1994) over the course of 1–2 weeks. In the present study, axial abdominal SERCA expression changed over a similar time course, with highest levels occurring approximately 2 weeks after ecdysis during intermoult. SERCA expression may also respond to the degree of muscular contractility, which is highest during intermoult; behavioural observations reveal that crustaceans cease routine locomotor activities around ecdysis.

Continued work will focus on the moult-related changes in expression of SERCA in other muscle types such as cardiac muscle, which is continuously active, and claw muscle, which atrophies in premoult. The probe described in the present study hybridized with lobster claw muscle (*Homarus americanus*; A. J. El Haj and M. G. Wheatly, unpublished data) and exhibited an identical pattern of expression during the moulting cycle even though the claw muscle exhibits a different pattern of intermittent growth. The role SERCA plays in the sequestration of Ca^{2+} during mass transit of Ca^{2+} across epithelial cells such as gill, antennal gland and hepatopancreas is being explored. Subcellular Ca^{2+} homeostasis will involve integration between SERCA, PMCA and the Na^{+}/Ca^{2+} exchanger; this equilibrium will be perturbed as transepithelial Ca^{2+} flux increases. Recently, we have cloned and sequenced a 2724 bp fragment of the crayfish PMCA and demonstrated that expression in gill epithelia is diametrically opposed to trends observed for SERCA, namely low during intermoult and premoult then increasing during postmoult to effect branchial net Ca^{2+} uptake. While it is somewhat premature to extrapolate the findings between different tissues, these preliminary data suggest inverse regulation of gene expression for Ca^{2+} pumps on internal and external membranes, confirming work in rat aortic endothelial cells in which SERCA was downregulated when PMCA was functionally overexpressed (Liu et al., 1996). This strongly suggests that pathways/mechanisms involved in Ca^{2+}-signalling systems are interdependently regulated by an unidentified mechanism.

The SERCA sequence from crayfish *Procambarus clarkii* axial abdominal muscle has been accepted by GenBank (Accession number AF025849).

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